Studies on *in-vivo* Antioxidant and Hepatoprotective Effects of Guava (*Pisidium guajava)* and Olive (*Olea uropaeal L.*)

Sherin I.Ibrahim*, Shaymaa Hussein[1] and Rehab A.Ghandour[2]

***Pharmacology Department, Faculty of Veterinary Medicine, Cairo University**

[1]Histology Department, Faculty of Veterinary Medicine, Cairo University

[2]Physiology Department, Faculty of Veterinary Medicine, Cairo University

*Corresponding author: E-mail:dr_sheren1982@yahoo.com

Tel: 01100431192

Address: 15 May City, Cairo, Egypt

Abstract

This study was applied to investigate the hepatoprotective activity of ethanolic extracts of edible Guava (*Pisidium guajava)* and Olive (*Olea uropaeal L*.) alone and against carbon tetrachloride (CCl_4) induced hepatic damage in rats. Oral administration of CCl_4 (1 ml/kg body wt. /day S/C) induced a chronic hepatotoxicity resulting elevated serum level of glutamate pyruvate transaminase (SGPT), glutamate oxaloacetate transaminase (SGOT) and alkaline phosphatase (ALP). The substantially elevated serum marker enzyme levels were restored towards normalization by the extracts treatment when administered at a dose of 500 mg/kg body wt. once daily for one month. The hepatic antioxidant status such as catalase (CAT), glutathione peroxidase (GPX), glutathione S. transferase (GST) and total antioxidant activity (TAC) levels are reduced in CCl_4 alone treated animals with subsequent increase in malondaldhyde lipid peroxidase (MDA). Administration of the extracts challenge restored the hepatic antioxidant status. Furthermore, histopathological studies confirmed the hepatoprotective effect. The findings suggested that ethanolic extracts of Guava (*Pisidium guajava)* and Olive (*Olea uropaeal L*.) exhibited a high liver protection against CCl_4 induced chronic hepatotoxicity in rats by restoring the liver antioxidant status.

Keywords: Antioxidant, Guava, *Pisidium guajav*, Olive, *Olea uropaeal L*. Carbon tetrachloride, Hepatoprotective effect.

Introduction

There is no doubt that reactive oxygen species (ROS) and free radicals such as superoxide onion (O_2), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) which formed in human by normal metabolic action play an important role in pathological changes in the liver **[1].** Their action is opposed the balanced system of antioxidant defenses, upsetting this unbalance causes oxidative stress, which lead to cell injury and death **[2].**Several endogenous protective mechanisms have been evolved to limit ROS and the damage caused by them **[3].**In case of excessive formation of ROS, additional protective mechanisms of dietary antioxidants may be of a great importance, therefore, many natural and artificial agents possessing antioxidative properties have been proposed to prevent and treat hepatic injury induced by oxidative stress **[4].**

Flavonoids and other organic compound which are widely distributed in fruits, vegetables and some herbs identified in recent years as antioxidants in various biological systems **[5].**

Liver damage induced by carbon tetrachloride (CCl_4) involves biotransformation of free radicals derivatives, increased lipid peroxidation and excessive cell death in liver tissue **[6].** This model of CCl4 induced liver injury has been widely used in new drug development for liver diseases.

Guava *(Pisidium guajava)* is a plant from family Myrtaceae. It extends throughout South America, European, Africa and Asia **[7].** *P. guajava* is widely used to treat gastrointestinal and respiratory disturbances and is used as an anti-inflammatory medicine; the water extract is used to reduce blood glucose level in diabetics **[8].** Leaves are applied on wounds, ulcers and for rheumatic pain **[9].**

Olive *(Olea europaeal L.)* from family Secoiridiod has been used as a folk remedy for combating fever, malaria and for lowering the blood pressure in animals **[10].** The major constituent of olive trees is oleuropein which has a potent antioxidant with anti-inflammatory properties and discovered by Bourquelot and Vintilescoin in 1908.Oleuropein has antimicrobial activity against viruses, bacteria, yeasts, fungi and molds **[11].**

The purpose of this study was to investigate the antioxidant and hepatoprotective effects of an ethanolic extracts of *pisidium guajava* and *olea europaea* L. alone and on cirrhosis development by Ccl_4 intoxication in rats.

Materials and methods

1. Plant material:

The whole plant of Guava (*Pisidium guajava)* and Olive (*Olea uropaeal L.*) were collected from field and market of Egypt. The plants are air dried in an oven at 40°C for 48 h. 250 gm. of dried powdered plant sample was extracted by one liter of ethanol 70% at 30°C for 48 h and filtering through whatman No. 4 filter paper. Then the plants were extracted by rotatory evaporation at 50°C till complete dryness occurs. The total extract was dissolved in water in a concentration of 500 mg/ml and stored at -20°C for further use **[12].**

2. Animals:

Healthy Swiss albino rats of approximately same age weighing about 150 g were used for the study. They were fed with standard diet and water *ad libitum* and housed in polypropylene cages under standard condition.

The animals were maintained according to the guidelines recommended by Animal Welfare Board and approved by our institutional ethics committee.

3. Ccl_4 induced hepatotoxicity and assessment of liver damage:

Experiment 1: The animals were divided into 3 groups of 5 animals each. Group 1 which served as normal control received saline (1 ml/day p.o.) for one month. Group II and Groups III received ethanolic extracts of *Pisidium guajava* and *Olea uropaeal L* (500 mg/kg body wt. P.o.) once daily, respectively for one month. The animals were scarified 24 h after the last treatment. The liver samples was excised and kept at 80°C for the determination of antioxidants.

Experiment 2: The animals were divided into 4 groups of 5 animals each. Group 1 which served as normal control received saline (1 ml/day p.o.) for one month. Group II, the positive control was given Ccl_4 in paraffin oil (1:1, 1 ml/kg body wt/day s/c) once daily for 7 consecutive days. Groups III and IV received ethanolic extracts of *Pisidium guajava* and *Olea uropaeal L*. 500 mg/kg body wt. P.o. respectively for one month then injected with Ccl_4 in paraffin oil (1:1, 1 ml/kg body wt/day s/c) once daily for 7 consecutive days. The animals were sacrificed 24 h after the last treatment of Ccl_4. Blood was collected. Allowed to clot and serum was separated by centrifugation at 2500 rpm for 15 min and biochemical investigations were carried out. A part of liver samples was excised and kept at 80°C for the determination of antioxidants and other part was fixed in 10% buffered formalin for histopathological assessment of liver damage.

4. Determination of hepatoprotective effect:

As a marker of hepatocytes necrosis, the activities of serum hepatic marker enzymes namely, serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT) and alkaline phosphatase (ALP) were assayed using assay kits (Span diagnostic, Surat).

5. Evaluation of antioxidant status:

Liver samples were dissected out and washed immediately with ice-cold saline to remove as much blood as possible. Liver homogenates (10% w/v) were prepared in cold PBS (50 mm, pH7) the homogenate was centrifuged at 5000 x g for 10 min in a centrifuge at 4°C, and after removal of cell debris, the supernatant was used for assay of catalase (CAT), glutathione peroxidase (GPX), glutathione s-transferees (GST), malondialdehyde lipid peroxidase (MDA) and total antioxidant activity (TAC) using assay kits (Span diagnostic, Surat).

6. Histopathological examination:

6.1. Light microscopy:
Samples fixed in 10% neutral buffered formalin, dehydrated in ascending grades of alcohol, cleared in benzene and methyl benzoate then embedded in paraffin wax. Paraffin blocks were sectioned at (3-5 µm thick) by rotatory microtome. Sections from each block were stained using the following methods: Harris haematoxylin and Eosin (H&E) stain for general tissue structure and Masson's trichrome stain for demonstration of collagenous and muscle fibers**.** Histochemical Study: Previous paraffin blocks sectioned and stained with periodic acid Schiff (PAS) for detection of neutral mucopolysaccharides.The fore mentioned stains were conducted as outlined by **[13, 14].**

6.2. Immunohistochemical study:

Deparaffinized sections autoclaved or incubated in 1% trypsin solution for antigen retrieval. After that, sections immersed in 0.3% hydrogen peroxide to block internal peroxidase, and in skimmed milk to block non-specific antibody binding. Primary antibodies were alpha smooth muscle actin (α-SMA) for smooth muscle. Biotinylated anti mouse or rabbit immunoglobulin G applied as secondary antibody. The sections incubated with peroxidase-labeled streptavidin and visualized in diaminobenzidine-tetrahydrochloride solution then counter staining done **[15].**

6.3. Evaluation of histological and histochemical observations:
Histological and Histochemical stained sections were examined using Leica Quin 500 analyser computer system (Leica Microsystems, Switzerland) in Faculty of Dentistry, Cairo University. The image analyser was calibrated automatically to convert the measurement units (pixels) produced by the image analyser program into actual micro meter units. Histological and Histochemical stains were measured as area percent in standard measuring frame in 5 fields from different slides in each group using magnification

(X400) by light microscopy transferred to the monitor's screen. The areas showing the positive histochemical stains reaction have chosen for evaluation, regardless the intensity of the staining. These areas masked by a blue binary colour to measure by the computer system**.** Mean value and standard deviation obtained for each specimen and statistically analyse.

7. Statistical analysis:

Data were presented as mean and standard deviation (SD).

When one –way ANOVA showed significant differences among groups. *Tukey's post hoc* test was used to determine the specific pairs of groups that were statistically different. A level of $p>0.05$ was considered statistically significant. Analysis was performed with the software SPSS version 16.0 (SPSS Inc, USA).

Result

1. Hepatoprotection:

The hepatoprotective effect of ethanolic extracts of *P.guajava* and *O.uropaeal L* on Ccl_4 induced hepatic injury in rats are shown on **Table 1**, chronic exposure to Ccl_4 revealed elevated liver function indices which as SGOT, SGPT and ALP compared to the normal set. The increased activity of serum marker enzymes may explain cell membrane breakdown and death **[16].** The SGOT, SGPT and ALP activities in CCl_4 treated rats were 181.40 ± 5.36, 87.04 ± 5.98 IU/L and 50.05 ± 2.2 KA respectively. The enzymes activities (SGOT, SGPT and ALP) were lowered by ethanolic extracts of *P.guajava L*. by 23%, 26% and 24% respectively and inhibited by 24%, 27% and 23%, respectively for ethanolic extract of *O.uropaeal L* compared with control Ccl_4 set.

2. Antioxidant status in liver:

A significant increase in antioxidant of CAT in liver tissues of rats between the normal set and groups treated with ethanolic extracts of *P.guajava* and *O.uropaeal L* without intoxication with Ccl_4. While there were insignificant changes in other antioxidant enzymes (**Table 2**).

A significant increase in level of MDA was found in the liver tissues of animals intoxicated with Ccl_4 group II when compared with normal set. Treatment with the extracts of *P.guajava* and *O.uropaeal L* in groups II and III resulted in significant lowering of MDA comparable with positive control set. (**Table 3**).

The activity of CAT, GPX, GST and TAC was observed to be decreased on Ccl_4 intoxicated group. Whereas treatment with the extracts of *P.guajava* and *O.uropaeal L* appeared to exert a beneficial effect since the hepatic antioxidant level is restored when compared to the normal group.(**Table3**).The augmentation in antioxidant enzymes activity is a sign of improve hepatic function.

3-Histopatholoical results:

Histopatholoical examination showed that liver tissues of rats in normal group exhibited normal hepatic plates between normal sinusoids around central vein and normal portal area while congestion in central vein and portal area and significant increase of non-healthy areas observed in Ccl_4 administrated group. Meanwhile, in *P.guajava* treated group showing less congestion in central vein and portal area and significant decrease of non-healthy areas. In *O.europaeal L* treated group revealed disappearance of congestion in central vein and portal area with significant dimension of non-healthy areas **(Table 4 & Plate 1)**.

Appearance of vacuoles observed in Ccl_4 administrated group while decreased number and size of these vacuoles in C: *P.guajava* and *O.europaeal L* treated groups, in addition dimension of non-healthy areas in *P.guajava* administrated group **(Plate2)**.

A significant increase in area % of collagen fibers recorded in Ccl_4 administrated group than other three groups and significant decrease in area % of collagen fibers in *P.guajava* and *O.europaeal L* administrated groups than Ccl_4 administrated group **(Table 4 & Plate 3).**

A significant decrease in area % of PAS (periodic acid-Schiff) observed in Ccl_4 administrated group than other groups**.** A significant increase in area % of PAS in *P.guajava* and *O.europaeal L* administrated groups were observed more than Ccl_4 administrated group **(Table 4 & Plate 4).** The PAS positive granules accumulations were seen in periportal hepatocytes.

A significant increase in area % of α-SMA immunohistochemistry were observed in Ccl_4 administrated group. Few α-SMA immune-expressions observed in normal group with a significant decrease in *P.guajava* and *O.europaeal* L administrated groups **(Table 4 & Plate 5).**

A significant increase in area % of α-SMA immunohistochemistry were observed in Ccl_4 administrated group. Few α-SMA immune-expression in normal group and moderate in *P.guajava* and *O.europaeal L* plus Ccl_4 administrated group **(Table 4 & Plate 5).**

Conclusion:

Thus it can be concluded that ethanolic extracts of *P.guajava* and *O.europaeal L* is able to confer protection against hepatotoxicity induced by Ccl_4 in rats, which might be through the antioxidant defense mechanism.

Discussion

In the present study the chronic administration of Ccl_4 induced hepatic injuries in rats which is commonly used model for hepatoprotective drug screening **[17].**

The experimental damage produced by Ccl_4 intoxication simulates many of the features of human liver fibrosis due to viral hepatitis **[18].**

The basis of hepatototoxicity of Ccl_4 lies in its metabolism in cytochrom P_{450} system forming two free radicals. The first metabolite is trichloromethyl radical (Ccl_3), forming covalent adducts with lipids in proteins; it can interact with O_2 to form the second metabolite which is trichloromethyl peroxy free radical (Ccl_3O_3), or can remove hydrogen atoms to form chloroform. This sequence of events leads to lipid peroxidation and liver injury **[19].**

The extent of hepatic damage is assessed by the level of increased cytoplasmic enzymes (SGPT, SGOT and ALP) and by histopathological examination **[20].**

Treatment with extracts of Guava (*Pisidium guajava)* and Olive (*Olea uropaeal L*.) lower serum transaminases indicating stabilization of plasma membrane as well as repair of hepatic injury. The elevated serum ALP activity was due to the intraheptic cholestasis **[21]** which was reduced in the extract treated animals. These effects are in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and regeneration of hepatocytes **[22].** Even pathological alteration in biliary flow is reflected by the enzyme ALP **[23].**

Administration of Ccl_4 alone decreased the antioxidant enzymes activity (CAT, SOD, GSH) which responsible for the increased lipid peroxidation measured as thiobarbituric acid reacting substance MDA, which lead to loss of membrane fluidity, membrane integrity, and final loss of cell functions of liver **[24]**, this injury in the hepatocytes alters their transport function and membrane permeability leading to the leakage of enzymes from the cell **[25].** The treatment of the extracts increased the hepatocytes SOD,

CAT activities and reduced lipid peroxidation MDA which could effectively prevent loss of membrane integrity and showed reduced transaminases activity in the serum of treated animals.

Reduced glutathione GSH plays a key role in the detoxification of reactive toxic metabolites ROS, liver necrosis is initiated when reserves of GSH are markedly depleted **[26].**

Histological studies were performed to provide direct evidence of hepatotoxicity of Ccl_4 and hepatoprotective effect of extracts. This complemented the results of liver enzyme studies. In which the free radicals (CCl3) of Ccl_4 metabolites are the main cause of liver injury. This is generated by its reductive metabolism by hepatic cytochrome P450 **[27].** The reactive intermediate metabolites cause lipid peroxidation and breakdown of cellular membranes **[28.29].** This explains the significant increase of area % of collagen fibers and non-healthy areas observed in Ccl_4 administrated group.

Congestion in liver in Ccl_4 administrated group was minimized in *P.guajava* administrated group and disappeared in *O.europaeal L* administrated group. This due to vasculo protector **[30].**

In the present study significant increase in area % PAS in *P.guajava* plus Ccl_4 administrated group and *O.europaeal L* administrated group than Ccl_4 administrated group. The periodic acid-Schiff (PAS) stain is useful for identifying glycogen **[31].** Glycogen depletion recorded in hepatotoxicity **[32].**

In our study, the immunohisto-chemical analyses of liver sections showed increase in area % of α-SMA immunohistochemistry in Ccl_4 administrated group. The increase in α-SMA release results from HSC (Hepatic stellate cells) activation due to liver damage. Following liver injury, HSCs show a reduction in vitamin A content and an increase in expression of α-SMA **[33,34].** α-SMA was performed to detect cell proliferation in both hepatocytes and non-hepatocytes in liver **[32].**

Our studty in *P. guajava* is in agreement with Koto-te-Nyjwa Ngbollua *et al*., **[35]** Who suggested that the aqueous extract of leaves of *P. guajava* possessed a hepatoprotective activity due to presence of secondary metabolites present in the plant especially triterpenoids derived acids (Guava coumaric, Ursolic,2 α-hydroxyurosolic, Maslinic, Asiatic, Jacoumaric. Isoneriucoumaric, Guajavanoic and Guavenoic acids). Our study also in agreement with Raksha Mishra *et al*., **[36]** who suggested that the bark of *P. guajava* revealed the presence of metabolites and compounds like alkaloids, glycosides, reducing sugar, phenolic compound, steroids, terpenoids, amino acid, flavonoids, tannins and saponin which show so many medicinal and pharmacological properties.

Our study in *O.europaea* is in agreement with Md. Yaseen K., *et al*., **[37]** who suggested that olive leaf has antioxidant properties associated with oleuropein, and also agreed with Ehsen H., *et al*., **[38]** Who studies the pharmacological activities and chemicalcomposition of the *olea europaea L*. leaf essential oils and suggested that *O. europaea* has *in vitro* antioxidant activity by using G.C.Ms analysis, and also our study in agreement with Muhammad A.H. *et al*., **[39]** who suggested that crude extracts and isolated components of O. europaea provide a reasonable support for its traditional uses like antioxidant, antidiabetic and anticancer. The phytochemical research carried out on O. europaea has led to the isolation of many classes of compounds like iridoids, secoiridoids, lignans, biophenols, flavonoids, flavone glycosides, isochromans and terpenoids which responsible for its pharmacological effects.

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Table 1: Effects of *P.guajava* and *O.europaeal L* ethanolic extracts on biochemical parameters of serum in rats.

Group	Treatment	SGOT`	SGPT	ALP
Group I	Normal	116.33± 6.94	55.32± 6.47	21.6± 2.37
Group II	Ccl_4 administrated group	181.40± 5.36	87.04± 5.98	50.05± 2.2
Group III	*P.guajava*	138.06± 4.9	64.47± 3.38	37.65± 3.41
Group IV	*O.europaeal L*	134.92± 4.06	63.48±2.95	38.41± 3.86

Table 2: Antioxidant effect of experimental extracts in normal rats.

Group	Treatment	Catalase U/GT	GPX U/GT	GST U/GT	MDA n mol/GT	TAC mM/L
Group I	Normal	0.522 ± 0.087 a	1099.02 ± 113.783 a	8.43 ± 1.22 a	10.88 ± 0.41 a	0.76 ± 0.03 a
Group II	*P.guajava*	0.717± 0.027 **b**	998.62 ± 197.82 a	7.32 ± 1.54 a	10.27 ± 0.59 a	0.83 ± 0.04 a
Group III	*O.europaeal L*	0.728 ± 0.075 **b**	906.11 ± 167.44 a	7.75 ± 1.68 a	10.38 ± 0.62 a	0.84 ± 0.06 a

Table 3: Antioxidant effect of experimental extracts in Ccl_4 induced hepatic injury in rats.

Group	Treatment	Catalase U/GT	GPX U/GT	GST U/GT	MDA n mol/GT	TAC mM/L
Group I	Normal	0.59 ± 0.25 a	1812.60 ± 155.24 a	8.84± 0.55 a	10.22± 0.34 a	0.66 ± 0.25 a
Group II	Ccl_4 only (control group)	0.33 ± 0.49 b	686.33 ± 84.79 b	5.29 ± 1.59 b	12.96 ± 0.38 b	0.35 ± 0.22 b
Group III	*P.guajava* + Ccl_4	0.57 ± 0.21 a	1640.57 ± 345.99 a	7.96 ± 1.67 a	10.18 ± 0.38 a	0.58 ± 0.18 a
Group IV	*O.europaeal L* + Ccl_4	0.62 ± 0.16 a	1681.19 ± 415.87 a	8.23 ± .48 a	10.10 ± 0.51 a	0.62 ± 0.05 a

Table 4: The histological, histochemical and immuno-histochemical observations of the experimental extracts in Ccl_4 induced hepatic injury in rats.

Group	Treatment	Area % of healthy tissue	Area % of collagen fibers	Area % of PAS	Area % of α-SMA
Group I	Normal	80.93 ± 1.224 **a**	0.890 ± 0.255 **a**	5.350 ± 1.120 **a**	6.050 ± 0.321 **a**
Group II	Ccl_4 only (control group)	50.730 ± 0.223 **b**	3.250 ± 0.112 **b**	2.535 ± 0.224 **b**	10.235 ± 2.325 **b**
Group III	*P.guajava* + Ccl_4	72.450 ± 3.479 **a**	0.745 ± 0.021 **a**	5.150 ±1.937 **a**	8.860 ± 2.771 **ab**
Group IV	*O.europaeal L* + Ccl4	77.865± 4.25 **a**	0.595 ± 0.191 **a**	6.800 ± 0.113 **a**	8.450 ± 1.428 **ab**

Values represented as means ± S.E. from 5 independent observations. Means with different letters in the same column are significant at $P<0.05$

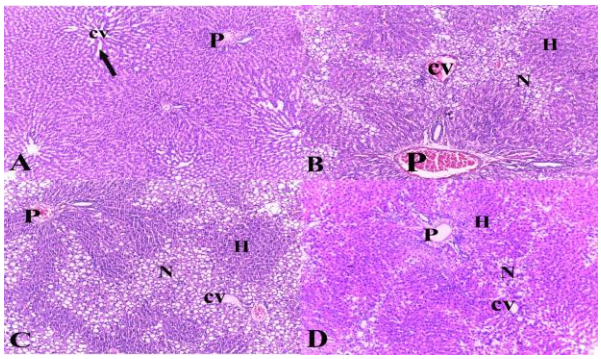

Plate 1: Photomicrograph of rat live A: normal group showing normal hepatic plates between sinusoids (arrow) around central vein (cv) and normal portal area (P). B: Ccl_4 administrated group showing congestion in central vein (cv) and portal area (P) and increase of non-healthy areas (N) between healthy areas (H). C: *P.guajava* plus Ccl_4 administrated group showing less congestion in central vein (cv) and portal area (P) and decrease of non-healthy areas (N). D: *O.europaeal L* plus Ccl_4 administrated group showing disappearance of congestion in central vein (cv) and portal area (P) with dimension of non-healthy areas (N) between healthy areas (H). H&E stain X100

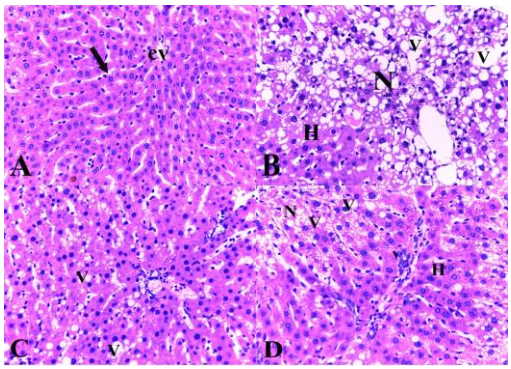

Plate 2: Photomicrograph of rat live A: normal group showing normal hepatic plates between sinusoids (arrow) around central vein (cv). B: Ccl_4 administrated group, showing increase vacuoles (V) in non-healthy areas (N) and decrease healthy areas (H). C: *P.guajava* plus Ccl_4 administrated group showing, decrease number and size of vacuoles (V). D: *O.europaeal L* plus Ccl_4 administrated group showing decrease number and size of vacuoles (V) with dimension of non-healthy areas (N) between healthy areas (H). H&E stain X400

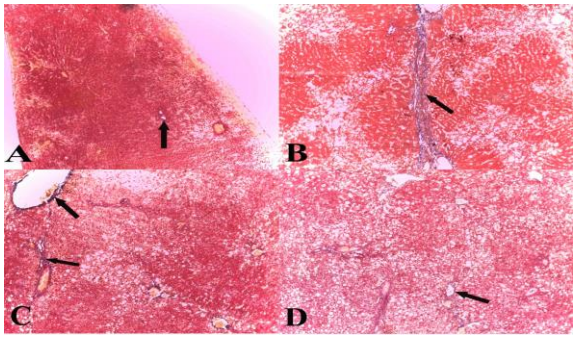

Plate 3: Photomicrograph of rat live A: normal group showing normal distribution of collagen fibers (arrow). B: Ccl_4 administrated group, showing increase collagen fibers (arrow). C: *P.guajava plus* Ccl_4 administrated group showing, decrease collagen fibers (arrow). D: *O.europaeal L* plus Ccl_4 administrated group showing decrease collagen fibers (H). Masson's trichrome stain X400 X100

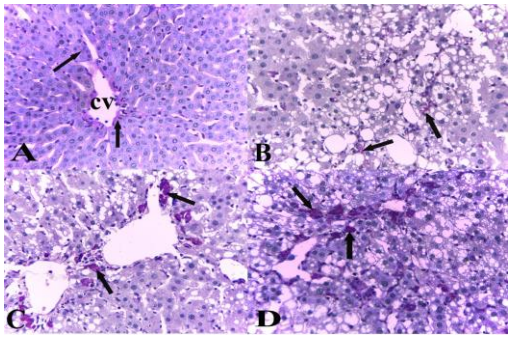

Plate 4: Photomicrograph of rat live A: normal group showing normal distribution of PAS positive granules (arrow). B: Ccl_4 administrated group, showing decrease PAS positive granules (arrow). C: *P.guajava* plus Ccl_4 administrated group showing, increase PAS positive granules (arrow). D: *O.europaeal L* plus Ccl_4 administrated group showing increase PAS positive granules. PAS stain X400

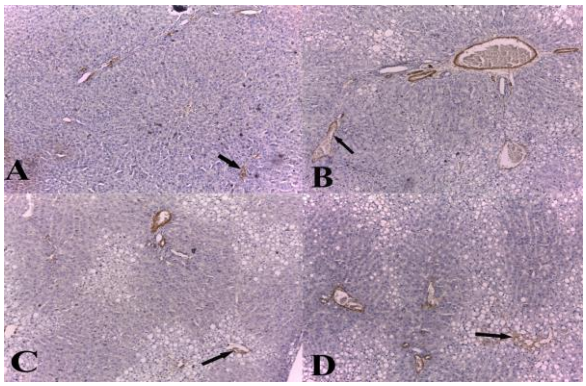

Plate5: Photomicrograph for α-SMA immunohistochemistry in rat liver X100 showing A: Few α-SMA immune expiration in normal group showing B: Increased in CCl4 administrated group. C: Moderate in *P.guajava* plus Ccl_4 administrated group. D: Moderate in *O.europaeal L* plus Ccl_4 administrated group.